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# BREAKTHROUGHS AND VIEWS

# Circadian Rhythm Biochemistry: From Protein Degradation to Sleep and Mating

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The behavior and physiology of most organisms show circadian, 24-h rhythmicity. Circadian oscillators are thought to be controlled by negative-feedback loops in clock genes expressed in organisms as simple as bacteria to mammals. Oscillating molecules that control their own expression in a circadian fashion seem to be very important for generating circadian rhythms in most organisms (1). In this minireview, the importance of protein degradation of circadian clock gene products as a molecular mechanism is summarized. We then discuss cell and tissue levels of clocks and the relationship between the molecular clock and sleep, and between the molecular clock and mating behavior.

## PROTEIN DEGRADATION AND CIRCADIAN CLOCK

The circadian expression in *Drosophila* of clock gene products, such as PER and TIM, is thought to be important for driving overt rhythms. The constitutive expression of *per* by the heat shock (2) or rhodopsin (3) promoters restores rhythmicity of the null allele of *per*, suggesting that *per* mRNA cycling may not be required for protein cycling or for locomotor rhythms. Furthermore, the constitutive expression of *tim* mRNA also supports protein cycling and behavioral rhythms in *tim* mutant flies (4). Sehgal *et al.* also showed that eliminating the oscillations of PER and TIM proteins by overexpression abrogated circadian rhythmicity. These data indicate that the circadian rhythmic expression of PER and TIM proteins is much more important than their rhythmic mRNA expression.

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Recent positional cloning has revealed that the *tau* locus (which shorten circadian rhythm) in hamsters is encoded by casein kinase Iε (5), a homology of the *Drosophila* clock gene double-time. The *double-time* gene product phosphorylates PER and causes protein degradation in *Drosophila* (6). Also in mammals, casein kinase 1ε (CK1ε) phosphorylates PER1, PER2 and PER3, then renders them unstable (7–9). Recent findings indicate that the human PER2 site phosphorylated by CK1ε is mutated in familial advanced sleep phase syndrome (10). This syndrome affects individuals who are "morning larks" in whom a 4-h advance of sleep, temperature, and melatonin rhythms suggests that sleep is under the control of the molecular circadian clock.

*Drosophila* TIM is degraded by a photic entrainment cue. In cultures, tyrosine phosphorylation-dependent TIM degradation is blocked by inhibitors of proteasome activity (11). These data suggest that the TIM degradation mechanism is involved through the ubiquitin–proteosome pathway. A new clock gene, shaggy/glycogen synthase kinase-3 (GSK-3) will be described soon, indicating a role for TIM phosphorylation (12). The data suggest that shaggy-dependent TIM phosphorylation increases PER/TIM heterodimerization or promotes the nuclear translocation of PER/TIM complexes in wild flies. TIM phosphorylation by shaggy may be different from protein degradation.

These data imply that the phosphorylation and protein degradation of clock gene products underlie the mechanism of circadian rhythm generation. The emergence of molecular components of the circadian clock sets the stage for elucidating the biochemical mechanisms in many diverse species from flies to humans.



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## NUMBER OF CLOCKS IN THE BODY

Although clock gene-based negative feedback loops between flies and mammals seem to be a side of unity (1, 13), diversity between the two species has recently become apparent. BMAL1 is a clock gene common to both *Drosophila* and mammals (1, 14). *Drosophila dclock* (jrk) mRNA is regulated in a circadian fashion, whereas *dBMAL1* (*cyc*) is not. Furthermore, dclock mRNA cycling is abolished and the values are troughs in *per* and *tim* mutants suggesting that *per* and *tim* genes can function as transcriptional activators of dclock mRNA expression in *Drosophila* (15). In contrast, mammalian cyclic *BMAL1* mRNA is expressed, but clock mRNA is not (16). Cyclic *BMAL1* mRNA expression is lost in *clock* mutant mice, suggesting that *BMAL1* mRNA is positively regulated by a CLOCK-BMAL1 feedback loop in the SCN of mammals (17). In this positive feedback loop, mammalian *BMAL1* appears to be the target circadian molecule instead of clock, as it is in the feedback loop of *Drosophila* (Figs. 1a and 1b) (15). Shearman *et al.* also showed that m*PER*2 is a positive regulator of the BMAL1 loop in the SCN (18).

Molecular differences between the central (SCN) and peripheral clocks in mammals are unknown, although both use the same set of genes. Okamura *et al.* have recently shown that the peripheral oscillator in cultured fibroblasts is identical to the central clock because the temporal expression and phase of mRNAs and proteins of several clock genes are identical in both the SCN and fibroblasts (19). However, the possibility between different molecular mechanisms between peripheral clocks and the central clock have been still remained. The situation *in vitro* does not always reflect that *in vivo.*

The anti-phasic circadian expression between *BMAL1* mRNA and r*PER*2 mRNA in several rat tissues has been reported (16, 20). The amplitudes of *BMAL1* and of r*PER*2 mRNA expression correlated among the various tissues, suggesting that the rhythmic expression of *per* 2 mRNA plays an important role in the circadian expression of mammalian *BMAL1* genes (16). However, the transcriptional regulation of *BMAL1* mRNA remains unknown. To examine whether or not circadian feedback loops driven by the CLOCK-BMAL1 heterodimer are required for the circadian expression of *BMAL1* mRNA *in vivo,* mRNA expression of the suprachiasmatic nucleus (SCN) and peripheral tissues of homozygous *Clock* mutant mice were examined by *in situ* hybridization and by Northern blotting (17). The results showed that in the SCN of *Clock* mutants, *BMAL1* mRNA did not oscillate significantly but was apparently expressed at low levels, while in the periphery, *BMAL1* mRNA levels were close to maximal in wild-type mice and did not show rhythmicity. Furthermore, daily expression of *mPer2* and *albumin site D-binding protein* (*DBP*) mRNA levels were severely blunted at trough values in both the periphery and the SCN. These data indicate that the circadian expression of *BMAL1* mRNA is affected by the CLOCK-BMAL1 induced transcriptional feedback loop in the SCN and in peripheral tissues through a different mechanism. Positive regulation in the central oscillator and negative regulation in the peripheral oscillator for *BMAL1* rhythmic expression might be

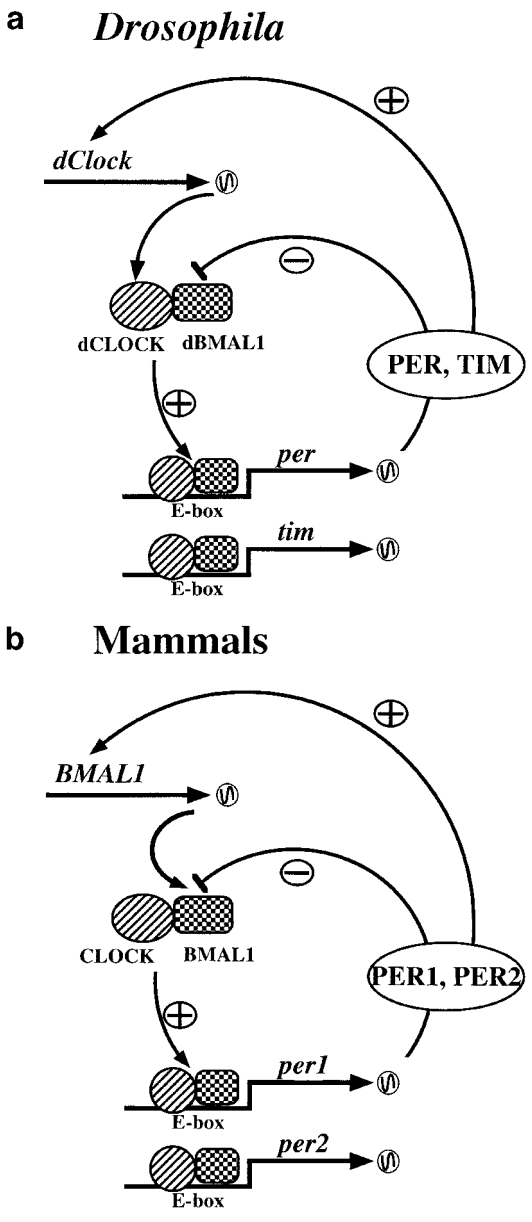


**FIG. 1.** Putative model of transcriptional feedback loops in *Drosophila* (a) and in SCN (b) of mammalian brain. Expression of mRNA is rhythmic for *dclock* in *Drosophila,* but *BMAL1* instead of *clock* shows rhythmic mRNA expression in mammals.

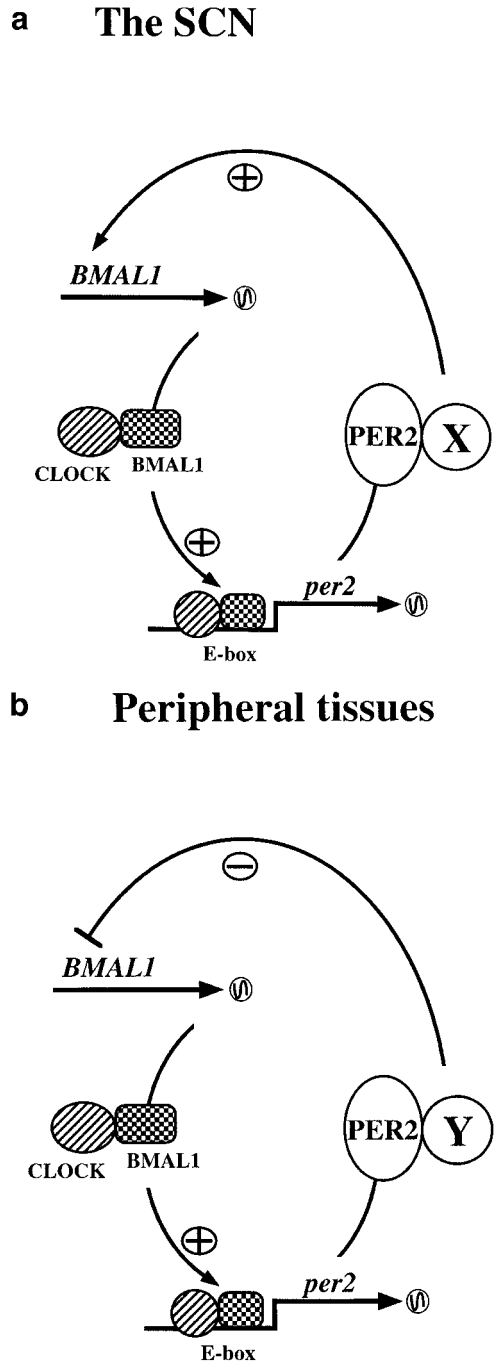


**FIG. 2.** Differential circadian feedback loops in central clock and peripheral clocks in mammals. Central clock (SCN, a) requires unknown positive regulator (X), but in peripheries (b) unknown negative regulator is needed to explain the differential BMAL1 mRNA expression in *clock* mutant mice.

required for the circadian expression of *BMAL1* mRNA in wild-type mice (Figs. 2a and 2b). Furthermore, the SCN seems to govern peripheral oscillators (21). Damping peripheral oscillators is supported by the pacemaker in the central oscillator (22). Restricted feeding studies have also suggested that the central oscillator and peripheral oscillators have different entrainment mechanisms with respect to food *in vivo* (23, 24).

One exciting discovery with regard to multiple clocks is the difference between right and left central oscillators (25). The concept of two coupled oscillators is not new (26). The concept is based on a phenomenon known as "splitting" that has been demonstrated in hamsters exposed to constant light, in which a single daily bout of locomotor activity dissociates into two components with different periods (27). This theoretical concept (28) is further supported by the findings that unilateral SCN lesions in split hamsters abolish behavioral splitting and produce a single bout of locomotor activity (29). Schwartz *et al.* showed that two putative oscillators under splitting correspond to the left and right sides of the SCN using the asymmetric expression of the *Per*1 and Bmal1 genes. Within *per* family of three genes, *Per*1 seems to be the most striking in terms of asymmetric expression. If so, then the left and right oscillator of the SCN will uncouple under exposure to constant light.

How the length of a day is understood from a circadian viewpoint remains unanswered. Measuring the length of the day indicates the season. To address this issue, the concept of morning and evening oscillators has been proposed (28). Daan *et al.* reported that *per1*/*cry1* expression in the SCN is accelerated by light and decelerated by darkness and thereby tracks dawn when day length changes (30). In contrast, *per2*/*cry2* expression is decelerated by light and accelerated by darkness and thereby tracks dusk. These data suggest that the morning and evening oscillators correspond to the *per1*/*cry1* and *per2*/*cry2* oscillators, respectively. The discovery of molecular components of circadian clocks will bring new insight into the physiological mechanisms such as how the length of days is recognized.

## SLEEP CONTROLLED BY CLOCK: LARKS OR OWLS

Whether the circadian and homeostatic processes of sleep are independent or whether they are interconnected at the molecular level has not been determined. However, recent progress indicates that mutation of the circadian clock gene affects several sleep phenotypes.

Albumin D-binding protein (DBP) is a PAR leucine zipper transcription factor that is expressed in a circadian fashion in the SCN and peripheral tissues such as the liver (31). The circadian rhythmicity of mice lacking DBP is shorter and less active, suggesting that

DBP is important for circadian output pathways. Recently, DBP-deficient mice (dbp−/−) have reduced ability to consolidate sleep episodes and the sleep–wake-dependent change of EEG delta power during non-REM sleep (32).

Individual differences in performance, morningness (lark-type)/eveningness (owl-type) are associated with physiological parameters such as body temperature (33), blood pressure and heart rate (34). Kleitman, in 1939, classified two distinct "morning" and "evening" types of human body temperature rhythm, one of which peaks early in the day and the other late (35). The circadian clock of an individual is believed to be important to the mechanism underlying morningness/eveningness (36), but substantial evidence has only recently started to accumulate.

Polymorphism of the 3′ flanking region of the *Clock* gene, which was originally identified as a mammalian circadian clock gene and a key player in circadian feedback loops (1), is associated with human morningness/eveningness preferences (37). If morningness/eveningness is related to the function of the *Clock* gene in mammals, an animal model should be able to create such lark/owl-type preferences. In fact, *Clock* homozygous mutant mice with a Jcl:ICR background that differs from the original BALB/c and c57/BL background has a clear evening preference for body temperature, spontaneous activity, and wake duration (38). The *clock* with a Jcl:ICR background was associated with clear REM sleep in the early morning (active phase), which was not evident in the isogonic control. The *clock* with a Jcl:ICR background did not show arrythmicity after exposing the mice to constant darkness for 3 months (data not shown). This model appears to be useful for molecular studies of the interaction between *clock* genes and sleep, but also for the timing of various diseases, such as myocardial infarction, sudden cardiac death, stroke, affective disorders, insomnia, and intolerance for shift work. The involvement of the circadian clock in the development and treatment of a wide variety of diseases is beginning to be recognized.

## *Drosophila* MATING BEHAVIOR ASSAYED BY "ARRANGED MARRIAGE"

Mating behavior in animals is the most important and fundamental process of selecting the best partner and to produce progeny. Some insects show daily rhythms in mating activity (39), which arises from interactions between fluctuating environmental stimuli and endogenous circadian clocks. The importance of light in mating behavior has been a key topic of study and discussion since 1946 (40). Genetic aspects of circadian clocks to mating behavior have been identified more recently (41). Mating frequency in *Dacus tryoni* is restricted to dusk, whereas that of its sibling species, *Dacus neohumeralis,* occurs in the middle of the day. The two species were crossed and the phase of F1 mating became as narrow as that of their parents, suggesting that the genetic mechanism of circadian mating is common between two species. Males of *Drosophila* extend and vibrate one wing vehemently at females, behavior which is referred to as "courtship." Although the relationship between the ultradian fluctuation of this wing vibration and *period* mutants is understood in detail (42), several *Drosophila* species show daily rhythms of male courtship behavior in LD cycles (43). However, the genetic mechanism that causes such fluctuation in mating rhythm on a daily basis remains totally unknown.

Our results using a new assay system indicate that *Drosophila melanogaster* wild-type displays a robust circadian rhythm in mating activity (44). After five males and five females were placed in a bottle for 20–30 min, we dissected out the female reproductive organs and calculated mating frequency as the percentage of the number of inseminated females over the number of dissected females. We call this assay system the "arranged marriage." Even in constant darkness (DD), mating rhythms are abolished in *period* or *timeless* mutant flies (*per01* and *tim01*) or in *disconnected* (*disco*) mutants that have a severe defect in the optic lobe and a lack of lateral neurons. The small lateral neurons are considered to be pacemakers for the locomotor rhythm of *Drosophila*.

The mating rhythms were lost when locomotor rhythm mutant females were paired with wild-type males, demonstrating that female mating activity is mainly governed by clock genes. To determine whether or not the *per* gene affects the reduction in mating activities at CT12, we measured mating activity using combinations of Canton-S males and both types of *per01* females in which PER is induced by heat shock. No differences in the mating activities of pairs between CT12 and CT18 were detected without heat shock or in the *per*01 mutant. However, mating activities at CT12 were significantly lower than those at CT18 after heat shock, suggesting that the induced PER protein causes a reduction in mating activity at CT12 and that arrhythmicity in the mating activity of the *per01* mutant is caused by the *per* mutation of female flies rather than by the genetic background of the mutants.

Furthermore, an antiphasic relationship in the circadian rhythms of mating activity was detected between *D. melanogaster* and their sibling species *D. simulans,* both of which are from Ogasawara Island

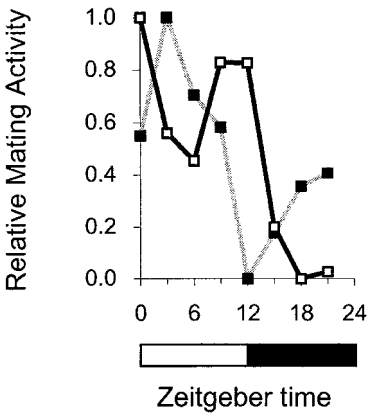


**FIG. 3.** Relative mating activity level in *D. melanogaster* (OGS-4, filled squares) and *D. simulans* (Ogasawara, open squares) in LD cycles. Relative mating activity is defined as follows: highest mating frequency = 1, and lowest mating frequency = 0. At ZT0, ZT9, and ZT12, mating activity levels of *D. simulans* were high, but those of *D. melanogaster* were median or low. When the activity level of *D. melanogaster* was high (ZT3), that of *D. simulans* was median.

(Fig. 3). The data suggested that female- and species-specific circadian rhythms in the mating activity of *Drosophila* cause reproductive isolation, which is an important factor in evolution. Understanding the temporal characteristics of mating behavior will bring new insight into the mechanisms of evolution.

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